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Abstract: Giant congenital naevi are pigmented childhood lesions that frequently lead to melanoma, the most aggressive skin cancer. The mechanisms underlying this malignancy are largely unknown, and there are no effective therapies. Here we describe a mouse model for giant congenital naevi and show that naevi and melanoma prominently express Sox10, a transcription factor crucial for the formation of melanocytes from the neural crest. Strikingly, Sox10 haploinsufficiency counteracts Nras(Q61K)-driven congenital naevus and melanoma formation without affecting the physiological functions of neural crest derivatives in the skin. Moreover, Sox10 is also crucial for the maintenance of neoplastic cells in vivo. In human patients, virtually all congenital naevi and melanomas are SOX10 positive. Furthermore, SOX10 silencing in human melanoma cells suppresses neural crest stem cell properties, counteracts proliferation and cell survival, and completely abolishes in vivo tumour formation. Thus, SOX10 represents a promising target for the treatment of congenital naevi and melanoma in human patients.

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Sox10 promotes the formation and maintenance of giant congenital nevus and melanoma

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Giant congenital nevi are pigmented childhood lesions that frequently lead to melanoma, the most aggressive skin cancer. The mechanisms underlying this malignancy are largely unknown, and effective therapies are missing. Here we describe a mouse model for giant congenital nevus and show that nevi and melanoma prominently express Sox10, a transcription factor crucial for the formation of melanocytes from the neural crest. Strikingly, *Sox10* haploinsufficiency counteracts *Nras*^{Q61K}–driven congenital nevus and melanoma formation without affecting physiological functions of neural crest derivatives in the skin. Moreover, Sox10 is also crucial for maintenance of neoplastic cells *in vivo*. In human patients, virtually all congenital nevi and melanomas are SOX10 positive. Furthermore, *SOX10* silencing in human melanoma cells suppresses neural crest stem cell properties, counteracts proliferation and cell survival, and completely abolishes *in vivo* tumor formation. Thus, SOX10 represents a promising target for treatment of congenital nevi and melanoma in human patients.

Human giant congenital nevi are associated with a high risk of melanoma. Mutations in *NRAS* are frequently found in human giant congenital nevi and melanomas, while *BRAF* mutations are usually not detected in giant congenital nevi[1-3]. Moreover, mutations in the *INK4a* locus that lead to inactivation of tumor suppressor genes are most common in human melanoma. In a melanoma mouse model, overexpression of the *Nras*^{Q61K} oncogene under the control of tyrosinase promoter, in combination with loss of *INK4a*, results in melanoma formation with a high penetrance (>90%) at around 6 months of age[4]. In contrast, *INK4a*$^{-/-}$ mice barely develop melanoma[5]. *Tyr::Nras*^{Q61K} expression is,

therefore, the key event underlying melanoma formation in the *Tyr::Nras*^{Q61K} *INKa*$^{-/-}$ mouse model. Intriguingly, morphological appearance and histological analysis of the skin of *Tyr::Nras*^{Q61K} mice showed some challenging similarities to human giant congenital nevus such as hyperpigmentation of the nevus nests close to the epidermis and location of nevus cells around and within hair follicles and other adnexal structures, such as sebaceous glands (Fig. 1). As in human patients, skin hyperpigmentation was apparent already at first postnatal week in *Tyr::Nras*^{Q61K} and *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ mice and was restricted to the dermis with virtually no epidermal involvement (Fig. 1d-f). Thus, *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ mice represent a model for melanoma arising from giant congenital nevi.

The transcription factor Sox10 is a key regulator of pigment cell formation during embryonic development and is expressed throughout all stages of melanocyte differentiation[6-9]. To investigate whether Sox10 is also expressed in pathological lesions associated with the melanocytic lineage, we first performed immunohistochemical analysis of human giant congenital nevi and revealed that SOX10 was highly expressed in all of the tested samples (n=17) (Fig. 2a,b, Supplementary Fig. S1a,b and Supplementary Table S1). Furthermore, extending studies by others[10-14], we analyzed expression of SOX10 in a large set of human primary melanomas and melanoma metastases[15]. We found that the vast majority of all melanoma samples displayed nuclear SOX10 staining (Fig. 2c,d). Of note, 100% of all primary melanomas were marked by SOX10 expression, with more than 90% of all cells being SOX10-positive in 85% of all samples tested (n=48). Of the metastatic samples, only 13% of the samples exhibited either very restricted or no nuclear SOX10 staining (n=130) (Fig. 2d). In

contrast, MITF, a transcription factor previously implicated in melanoma biology[16], was not expressed in all primary melanoma samples and, in particular, absent in 11% of tumor biopsies exhibiting dominant SOX10 expression (Supplementary Fig. S1c,d). Therefore, SOX10 might serve as a more sensitive marker for human giant congenital nevus and melanoma than other currently used markers including MITF.

In analogy to the human situation, dermal lesions reminiscent of giant congenital nevi exhibited broad Sox10 expression in *Tyr::Nras*^{Q61K} and *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ mice from early postnatal stages onwards (Fig. 2e). Moreover, Sox10 was prominently expressed in tumors generated in *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ mice at 6 months of age (Fig. 2f). To address whether expression of Sox10 might be regulated by oncogenic *Nras*^{Q61K}, we purified melanocytic cells from the skin of one month old wild type and *Tyr::Nras*^{Q61K} mice by fluorescent activated cell sorting (FACS), using the lineage markers MelanA and c-Kit for cell surface labeling (Fig. 2g). Quantitative real time PCR analysis of the resulting cell fractions demonstrated that Sox10 expression was significantly increased in melanoblasts from *Tyr::Nras*^{Q61K} mice as compared to control melanoblasts, revealing *Nras*^{Q61K}-mediated Sox10 activation (Fig. 2h). The combined findings strongly suggest a central role of SOX10 in both giant congenital nevus and melanoma formation in human and mice.

During development, melanocytes arise from the neural crest, a transient embryonic structure containing multipotent neural crest stem cells (NCSCs). Absence of Sox10 affects NCSC survival and leads, therefore, to a loss of neural crest derivatives, including melanocytes[6, 7, 17]. In mice heterozygous for *Sox10* null mutations, however,

most neural crest derivatives are properly generated during development, although neural crest stem and progenitor cell maintenance, proliferation, and fate decisions are impaired[6, 9, 18-22]. In particular, both in human and mice, *Sox10* haploinsufficiency is compatible with normal melanocyte specification and differentiation, but interferes with the generation of proper pigment cell numbers during development, leading to characteristic pigmentation defects at birth. In the adult, however, physiological pigmentation is not affected by monoallelic Sox10 expression.

Based on this knowledge, we reasoned that *Sox10* haploinsufficiency might possibly interfere with aberrant melanocytic cell production during the generation of giant nevi and melanoma without disturbing normal cell function. To test this hypothesis, we made use of $Sox10^{LacZ/+}$ mutant mice, in which the complete open reading frame of *Sox10* has been replaced by *LacZ* sequences[6] (Fig. 3a). Strikingly, in $Tyr::Nras^{Q61K}$ and $Tyr::Nras^{Q61K}$ $INK4a^{-/-}$ mice, *Sox10* haploinsufficiency counteracted $Nras^{Q61K}$–dependent hyperpigmentation, as evident by the morphological appearance of snout, paws, and other skin areas (Fig. 3a,b and Supplementary Fig. S2a). The loss of hyperpigmentation in $Tyr::Nras^{Q61K}$ $INK4a^{-/-}$ $Sox10^{LacZ/+}$ mice became particularly apparent upon shaving of the back skin of the animals (Fig. 3b). These data were confirmed by histological analysis of skin sections. While virtually all hair follicles (HFs) were associated with ectopic pigmentation in $Tyr::Nras^{Q61K}$ and $Tyr::Nras^{Q61K}$ $INK4a^{-/-}$ mice (95±5 and 94±6%, respectively), *Sox10* heterozygosity efficiently reverted this phenotype to an almost normal pigmentation pattern (HFs with ectopic pigmentation: 8±2% and 9±2%, respectively) (Fig. 3c,d). Likewise, the number of dermal cells positive for the melanocytic markers S100 or Dct[23-25] was massively

reduced due to *Sox10* haploinsufficiency (Fig. 3c and Supplementary Fig. S2c,d). *Tyr::Nras*^{Q61K} *Sox10*$^{LacZ/+}$ and *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ *Sox10*$^{LacZ/+}$ mice displayed pigment cells in HF bulbs and no precocious hair graying, however, revealing normal generation of melanocytes in these animals (Supplementary Fig. S2a,b).

To assess whether *Sox10* haploinsufficiency not only influences congenital nevus, but also subsequent melanoma formation, *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ *Sox10*$^{LacZ/+}$ mutant mice were monitored for several months, together with *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ control animals. Whereas 100% of the control mice developed macroscopically visible melanomas[4], skin examination of *Tyr::Nras*^{Q61K} *INK4a*$^{-/-}$ *Sox10*$^{LacZ/+}$ mice at 6 months of age revealed no morphological signs of primary melanoma formation (n=18) (Fig. 3e,f and Supplementary Fig. S2b). These findings demonstrate efficient inhibition of melanoma formation by *Sox10* haploinsufficiency in mice.

During embryonic development, Sox10 regulates the balance between NCSC maintenance and multilineage differentiation[17, 26]. In particular, *Sox10* haploinsufficiency counteracts maintenance and expansion of proliferative progenitor cells in different neural crest target structures[27, 28]. Therefore, to study the mechanisms underlying Sox10-dependent congenital nevus and melanoma formation, we addressed whether Sox10 haploinsufficiency affects cell proliferation by quantifying the number of Dct-positive cells expressing the proliferation marker Ki67 in wild type, *Tyr::Nras*^{Q61K}, and *Tyr::Nras*^{Q61K} *Sox10*$^{LacZ/+}$ mice (Fig. 3g-j). To this end, cell proliferation was assessed in melanocytic cells localized to HFs because this cellular compartment was present in all three genotypes. In the bulb area with differentiated

melanocytes (Fig. 3i,j) and in particular in the bulge area containing undifferentiated cells of the melanocyte lineage (Fig. 3g,h), expression of *Nras*^{Q61K} led to a highly significant increase in the number of proliferative Dct-positive cells. In *Tyr::Nras*^{Q61K} *Sox10*$^{LacZ/+}$ mice, however, proliferation was reduced to almost control levels (Fig. 3g-j). These experiments demonstrate that one of the main antagonistic effects of *Sox10* haploinsufficiency on tumorigenesis is to counteract *Nras*^{Q61K}-induced hyperproliferation.

To assess the importance of Sox10 functions for the maintenance of already established lesions, we used *Tyr-CreERT2* mice to conditionally induce *Sox10* heterozygosity in two months old *Tyr::Nras*^{Q61K} mice carrying a *Sox10*^{fl} allele[29] (Fig. 4a,b). To achieve inducible Cre-dependent recombination and thus loss of Sox10 expression from the *Sox10*^{fl} allele, mice were injected with tamoxifen at two months of age, followed by their analysis at 12 month (Fig. 4a). Intriguingly, hyperpigmentation was efficiently reverted in the back skin, snout and paws of these mice (Fig.4b). Moreover, histological analysis of skin sections showed that induced *Sox10* haploinsufficiency resulted in an almost complete disappearance of ectopic dermal cells positive for pigment or Dct (Fig. 4c). Indeed, while 96 ± 1% of all HFs were associated with ectopic Dct-positive cells in *Tyr::Nras*^{Q61K} *Sox10*$^{fl/+}$ mice, this number was reduced to 10 ± 3% in *Tyr::Nras*^{Q61K} *Sox10*$^{fl/+}$*Tyr-CreERT2* mice upon tamoxifen-induced Cre-mediated recombination (Fig. 4d). Thus, Sox10 is required not only for the formation but also for the maintenance of pre-melanoma lesions.

To address the relevance of these findings for human melanoma, we assessed whether in analogy to the mouse model, decreasing levels of SOX10 in human melanoma cell lines would interfere with melanoma maintenance. To this end, we performed RNA interference (RNAi)-mediated silencing of *SOX10* in human melanoma cell lines[30]. In two distinct cell lines, *SOX10* shRNA resulted in efficient downregulation of SOX10 expression, as revealed both by Western blot analysis and immunocytochemistry (Supplementary Fig. S3). The capacity to form clones consisting of two or more cells was significantly reduced upon *SOX10* knockdown (Fig. 4e; clonogenicity of cells expressing control shRNA, 27 ± 2%; *SOX10* shRNA, 4 ± 1%). To investigate *in vivo* tumor formation, immunocompromized mice were injected subcutaneously with melanoma cells expressing either *SOX10* or control shRNA. After 8 weeks, control cells consistently produced large, macroscopically evident tumors *in vivo* (11 out of 14 injections; Fig. 4f). In striking contrast, none of the subcutaneous injections with *SOX10* shRNA-expressing melanoma cells (0/16) led to tumor formation, even when the mice were kept for an additional 6 weeks. These data demonstrate that silencing of *SOX10* very effectively blocks tumorigenesis by human melanoma cells *in vivo*.

To address the mechanisms by which *SOX10* controls human melanoma formation, we performed a comparative genome-wide transcriptome analysis of control vs. *SOX10* shRNA-expressing melanoma cells 48 and 96 hours after RNAi-mediated silencing. Cluster analysis of transcriptionally regulated genes indicated that reducing SOX10 levels promotes apoptosis, cell cycle exit, and mesectodermal differentiation (Fig. 4g and Supplementary Fig. 4a). These data were confirmed by measuring changes in expression levels of 233 genes 48 and 96 hours after *SOX10* shRNA treatment, using

quantitative real time PCR (Fig. 4h,i, Supplementary Fig. 4b-f). Several genes encoding apoptosis-control factors were dysregulated upon silencing of *SOX10* (Fig. 4h). For instance, the anti-apoptotic factor BCL2 was significantly downregulated, while pro-apoptotic factors such as CASP1 and multiple TNF pathway components were highly upregulated in *SOX10* knockdown cells. Moreover, RNAi-mediated silencing of *SOX10* led to highly reduced expression of almost all genes tested that encode cyclins and cyclin dependent kinases (CDKs), which trigger progression through the cell cycle (Fig. 4i and Supplementary Fig. 4d). Thus, in human melanoma cells, SOX10 controls multiple genes associated with cell survival and proliferation.

To functionally demonstrate the cell death-promoting effect of *SOX10* silencing, we quantified the number of Annexin V-positive cells using FACS analysis (Fig. 5a-c and Supplementary Fig. 5a-d). This method revealed that in both human melanoma cell lines tested, the number of dying cells was significantly increased 48 and 96 hours after *SOX10* shRNA treatment of the cells (Fig. 5c). Likewise, expression of *SOX10* shRNA led to a more than 9 fold increase in the percentage of apoptotic cells positive for activated Caspase 3 (Supplementary Fig. 5c,d). Together, these experiments revealed an important role of SOX10 in supporting the survival of melanoma cells. To address the function of SOX10 in cell cycle control, we analyzed the cell cycle profile in control vs. *SOX10* shRNA-expressing human melanoma cells. These experiments demonstrated decreased cell cycle progression upon *SOX10* knock down (Fig. 5d-f and Supplementary Fig. 5e-j). Therefore, SOX10 is required for the maintenance of proliferative melanoma cells. Finally, as shown by immunocytological assays, loss of SOX10 promoted the formation of large, myofibroblast-like cells and expression of

SOX9, which in neural crest development marks mesectodermal progenitor cells[28] (Fig. 5g,j). In addition, the vast majority of *SOX10* shRNA-expressing melanoma cells lost expression of the melanoma marker HMB45/MART-1 (Fig. 5h,k). These findings are highly reminiscent of the effects seen upon *Sox10* loss of function in normal NCSCs, which include aberrant fate decisions, altered proliferation and survival, and increased differentiation into the mesectodermal lineages[17, 28]. Thus, *SOX10* shRNA expression in human melanoma cells appears to interfere with NCSC properties. In support of this, *SOX10* inactivation led to a drastic reduction in the number of cells expressing the NCSC marker CD271 ($p75^{NTR}$) (Fig. 5i,l). Intriguingly, this marker has been associated before with cells implicated in melanoma initiation and propagation[10, 31], which offers a plausible explanation for the anti-tumorigenic effect of *SOX10* silencing.

In this study, we demonstrate intriguing similarities between the *Tyr::*$Nras^{Q61K}$ mouse model and human melanomas arising in giant congenital nevi. These nevi often carry *Nras* mutations and are associated with a dermal origin of the malignant melanocytic tumor cell population. Although melanoma formation in this model implicates the loss of tumor suppressor genes encoded by *INK4a*, the key event required for nevus formation and tumorigenesis is activation of $Nras^{Q61K}$ associated with expression of the transcription factor Sox10. Intriguingly, *Sox10* haploinsufficiency fully prevents $Nras^{Q61K}$–driven formation of premalignant lesions and tumors. In human melanoma cells, suppression of SOX10 counteracts melanoma formation by interfering with NCSC features and decreasing the number of CD271-positive tumor-initiating cells. Based on our data, SOX10 levels might represent a molecular target to suppress giant congenital

nevi and their malignant transformation and to interfere with expansion of established melanomas.

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Author Contributions

O.S., P.C., and L.S. designed the experiments, O.S., P.C., D.Z., S.S., L.H., G.C., S.C., and J.B., performed the experiments, and O.S., P.C., M.O., D.M., R.D., Y.B., P.C. and L.S. analyzed the data. R.D. provided human samples of congenital nevus. D.M. and H.M. provided tissue microarrays of primary and metastatic melanoma samples. M.W. provided *Sox10*$^{LacZ/+}$ and *Sox10*$^{fl/+}$ mice, F.B. provided *Tyr::Nras*^{Q61K} mice. O.S. and L.S. wrote the manuscript.

Figure 1. Histological analysis of skin of *Tyr::Nras^{Q61K}* mice reveals lesions similar to human giant congenital nevus. a, Clinical features of congenital giant hairy nevus from human patient (girl, 6 months of age). **b**, Clinical features of giant congenital nevus (bathing trunk nevus) from human patient (girl, 12 years of age). **c**, Histological appearance of human giant congenital nevus. Note the lack of epidermal involvement. Scale bar, 50 μm. **d**, Picture of representative WT and *Tyr:: Nras^{Q61K}* mice at postnatal day 9. **e**, Pictures of snouts and paws of WT and *Tyr:: Nras^{Q61K}* mice. Note hyperpigmentation of the skin of *Tyr:: Nras^{Q61K}* mice as compared to WT mice. Scale bars, 2 mm. **f**, Histological appearance of skin from *Tyr:: Nras^{Q61K}* mice. Scale bar, 50 μm. H&E, haematoxilin and eosin, P9, postnatal day 9, SG, sebaceous gland, WT, wild type.

Figure 2. SOX10 expression is a reliable marker for giant congenital nevi, primary melanoma and melanoma metastases in *Tyr::Nras^{Q61K}INK4a$^{-/-}$* mice and in human patients. a, Immunostaining for SOX10 (nuclear, red) of skin from a human patient (girl, 6 months of age). Left panel, normal skin with SOX10-positive melanocytes; right panel, giant congenital nevus from the same patient. **b**, 17 samples of giant congenital nevi from different human patients were divided into three subgroups based on the size of the congenital nevi: group I (1.5 to 10 cm), group II (11 to 20 cm) and group III (21 to 40 cm). Nuclear SOX10 staining (red) was detected in all 17 samples analyzed independently of their size. **c**, **d**, Immunostaining for SOX10 in human melanomas. One primary and two different metastatic TMA (tissue microarray) were scored based on the percentage of SOX10-positive nuclear staining in melanoma cells (arrows). Samples were divided into four groups with expression in less than 10% of tumor cells (group I),

from 10 to 50 % (group II), from 50 to 90% (group III), and in more than 90% (group IV) of all tumor cells. Insets show high magnification views; n=178 melanomas. **e**, **f**, Sox10 expression (red) was also detected in giant congenital nevi (mouse at postnatal day 9) and melanoma (6 months old mouse) in *Tyr::Nras^{Q61K}INK4a$^{-/-}$* mouse model. **g**, **h**, Sox10 is upregulated upon expression of *Nras^{Q61K}* oncogene. **g**, Fluorescence histogram of gated cells of the melanocytic lineage from the trunk skin of one month old *WT* and *Tyr::Nras^{Q61K}* mice stained with a combination of MelanA and c-Kit antibodies. **h**, Cells from the MelanA/c-Kit double positive fraction were used for RNA isolation and subsequent quantitative real-time PCR analysis of Sox10 expression. n = 3 independent experiments. Data are presented as the mean fold change ± s.d. and are normalized to the control. BF, bright field, HF, hair follicle; SG, sebaceous gland. Scale bars, 50 μm.

Figure 3. *Sox10* haploinsufficiency counteracts *Tyr::Nras^{Q61K}*–mediated hyperpigmentation and melanoma formation by counteracting *Nras^{Q61K}*–dependent proliferation. a, Experimental strategy used to analyze the effect of *Sox10* haploinsufficiency in *Tyr::Nras^{Q61K}* and *Tyr::Nras^{Q61K} INK4a$^{-/-}$* mice. **b**, Pictures of two representative mice at 5 months of age show no skin hyperpigmentation in *Tyr::Nras^{Q61K}INK4a$^{-/-}$Sox10$^{LacZ/+}$* mice. Scale bars, 5 mm. **c,** H&E staining (left panels) and immunostaining for S100 (right panels) of skin sections from *Tyr::Nras^{Q61K}* and *Tyr::Nras^{Q61K}Sox10$^{LacZ/+}$* mice. Scale bars, 20 μm. **d**, Quantification of percentage of hair follicles (HFs) displaying ectopic pigmentation. Data are presented as mean ± s.d. (n=300 HFs quantified from three different mice for each genotype). **e**, Photograph showing pigmented melanoma (black arrows) in 6 month old *Tyr::Nras^{Q61K} INK4a$^{-/-}$*

mouse (left) and melanoma-free *Tyr::Nras^{Q61K} INK4a$^{-/-}$Sox10$^{LacZ/+}$* littermate mouse (right). To demonstrate presence of melanoma, mice with light coat color were used for this picture. Note that there is no hair greying in mice of both genotypes (Supplementary Fig. S2). **f**, Graph showing percentage of melanoma-free *Tyr::Nras^{Q61K} INK4a$^{-/-}$* (n=23 mice) and *Tyr::Nras^{Q61K} INK4a$^{-/-}$ Sox10$^{LacZ/+}$* (n=18 mice) mice at 7 months of age. **g**, **i**, Immunostaining for Dct (green) and Ki67 (red) showing proliferation of cells of the melanocytic lineage in the upper part of the HF (**g**) and in the HF bulb (**i**) in WT, *Tyr::Nras^{Q61K}* and *Tyr::Nras^{Q61K}Sox10$^{LacZ/+}$* mice (insets show high magnification views). Scale bars, 20 μm. **h**, Quantification of percentage of HFs containing at least one Ki67-positive Dct-expressing cell in the upper part of HF. Data are presented as mean ± s.d. (n=136, 128, 128 HFs counted from three different mice of WT, *Tyr::Nras^{Q61K}* and *Tyr::Nras^{Q61K}Sox10$^{LacZ/+}$* genotype, respectively). **j**, Quantification of percentage of HFs containing at least one Ki67-positive Dct-expressing cell in the HF bulb. Data are mean ± s.d. (n=212, 227, 251 from three different mice of WT, *Tyr::Nras^{Q61K}* and *Tyr::Nras^{Q61K} Sox10$^{LacZ/+}$* mice respectively). P9, postnatal day 9; HF, hair follicle; SG, sebaceous gland.

Figure 4. Sox10 function is crucial for the maintenance of already established melanocytic lesions in *Tyr::Nras^{Q61K}* mice and is required for proliferation and survival of cells with tumorigenic potential in human melanoma cell lines. a, Experimental strategy used to analyze the effect of *Sox10* haploinsufficiency in 2 months old *Tyr::Nras^{Q61K}* mice. **b**, Pictures of two representative mice 10 months after tamoxifen injections show no skin hyperpigmentation in *Tyr::Nras^{Q61K}Sox10$^{fl/+}$Tyr-CreERT2* mice. Scale bars, 5 mm. **c**, H&E staining and immunostaining for Dct (green)

of skin sections from *Tyr::Nras^{Q61K}Sox10$^{fl/+}$* and *Tyr::Nras^{Q61K}Sox10$^{fl/+}$Tyr-CreERT2* mice. Scale bars, 20 μm. **d**, Quantification of percentage of HF with ectopic Dct-expressing cells. Data are presented as mean± s.d. (n=394 and 400 HFs counted from three mice of each genotype, *Tyr::Nras^{Q61K}Sox10$^{fl/+}$* and *Tyr::Nras^{Q61K}Sox10$^{fl/+}$Tyr-CreERT2*, respectively). **e,** Colony formation by human melanoma cells upon lentiviral shRNA-mediated knockdown of *SOX10* (left, control; right, *SOX10* shRNA). Plates were stained with crystal violet to visualize colonies. Scale bars, 200 μm. **f**, Frequency of tumor formation in immunodeficient NOD/SCID or Nude mice upon subcutaneous injections of melanoma cell lines M010817 and M070302 expressing either *SOX10* shRNA or scrambled control shRNA. **g**, Heat map of mRNA expression upon *SOX10* KD after 48 and 96 hours in M010817 cell line. The signature used in the heat map was compiled from Affymetrix probe sets related to genes involved in apoptosis (61 probe sets), cell cycle (127 probe sets), melanocytic differentiation (46 probe sets) and mesectodermal differentiation (59 probe sets). **h-i**, mRNA expression of genes involved in apoptosis and cell cycle 96 hours after *SOX10* knockdown. n = 3 independent experiments. Data are presented as the mean fold change ± s.d. and are normalized to the control. HF, hair follicle; SG, sebaceous gland; P0, postnatal day 0; mo, months.

Figure 5. SOX10 is required for proliferation and survival of cells with tumorigenic potential in human melanoma cell lines and *SOX10* knockdown induces fate switch in melanoma cells. a-c, FACS analysis and quantification of Annexin V-positive cells upon *SOX10* KD after 48 and 96 hours in M010817 cell line. For apoptosis analysis, GFP-positive cells were gated (**a**) and Annexin V-positive cells were analyzed after 48 hours (**b**, left panel) and 96 hours (**b**, right panel) of *SOX10*

knockdown. **c**, Quantification of Annexin V-positive cells in GFP-expressing fractions in M010817 and A375 cell lines upon *SOX10* KD after 48 and 96 hours. Data are presented as mean± s.d. n=3 of independent experiments. These data show that *SOX10* knockdown influences survival of melanoma cells. **d-f,** FACS analysis of cell cycle profile in M010817 cell line upon SOX10 knockdown after 96 hours. GFP-positive cells were gated (**d**) and subjected further to PI selection gating. Resulting FACS profiles of M010817 control (**e**, left panel) and M010817 *SOX10* shRNA (**e**, right panel) after 96 hours of electroporation are presented. **f**, Quantification of ratio of cells in G_1 phase to cells in S plus G_2 phases upon SOX10 KD in M010817 and A375 cell lines are presented. Data are presented as mean ± s.d. n=3 of independent experiments. These data show that *SOX10* knockdown results in cell cycle arrest in melanoma cells. **g**, Immunostaining for SOX9 (red) combined with GFP signal (green) showing that melanoma cells acquire SOX9 expression upon knockdown of *SOX10*. **h**, Immunostaining for melanoma markers HMB45/MART-1 (red) combined with GFP signal (green) showing the reduction of HMB45/MART-1 expression in *SOX10* knockdown cells. **i**, Immunostaining for CD271 ($p75^{NTR}$) (green) and SOX10 (red) combined with GFP signal (white) in cells electroporated with SOX10 shRNA demonstrating loss of CD271 ($p75^{NTR}$) positive cells in GFP-expressing cells. **j,** Representative pictures of M010817 cells electroporated with control scr shRNA (**j**, left panel) and *SOX10* shRNA (**j**, right panel) demonstrating the change in morphology and smooth muscle-like cell appearance upon *SOX10* knockdown. **k-l**, Quantification of HMB45/MART-1-positive cells (**k**) and CD271 ($p75^{NTR}$)-positive cells (**l**) upon *SOX10* KD after 96 hours in M010817 cell line. n = 3 independent experiments. Data are represented as mean ± s.d**.** Cntl, control. Scale bars, 20 μm.

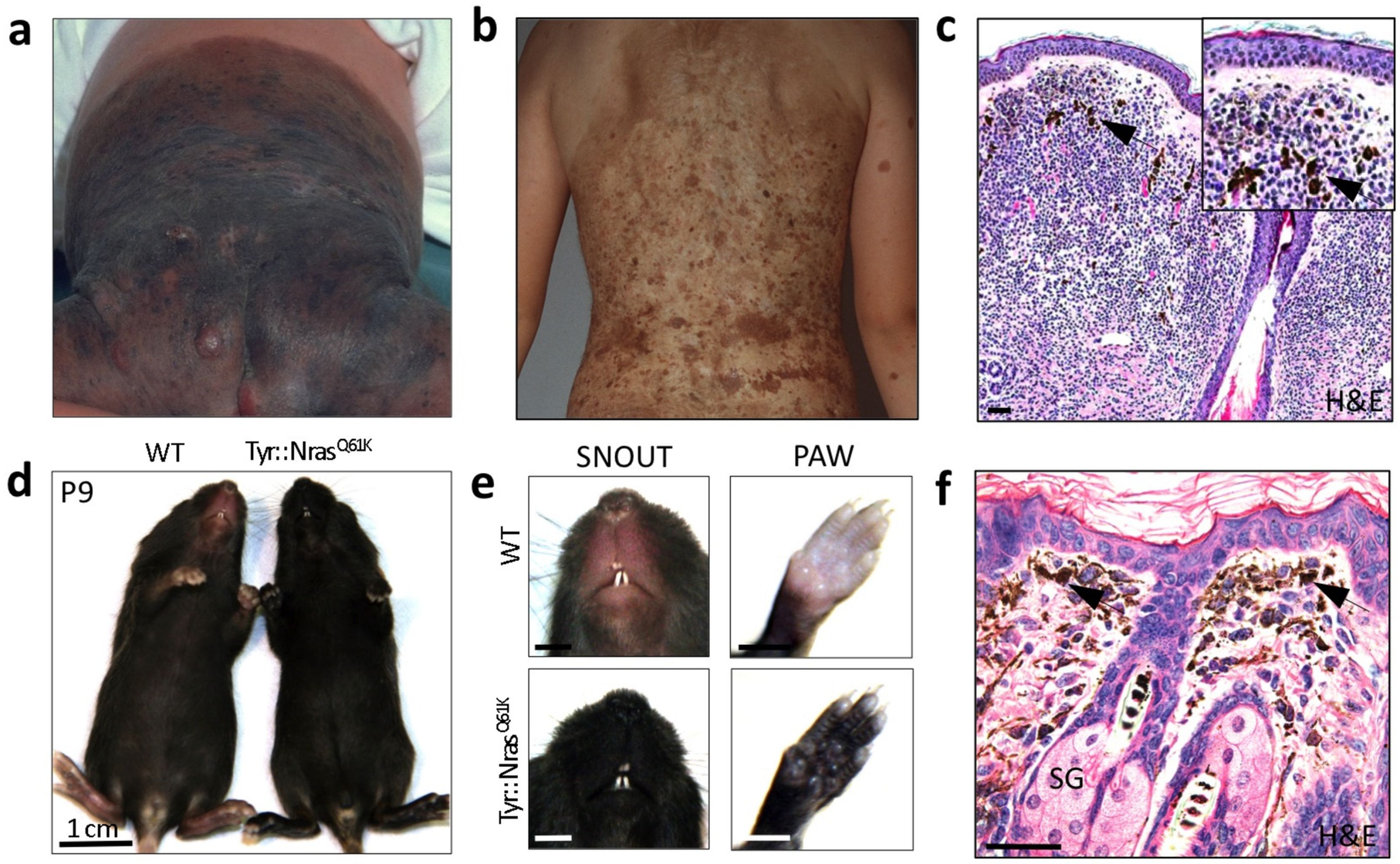
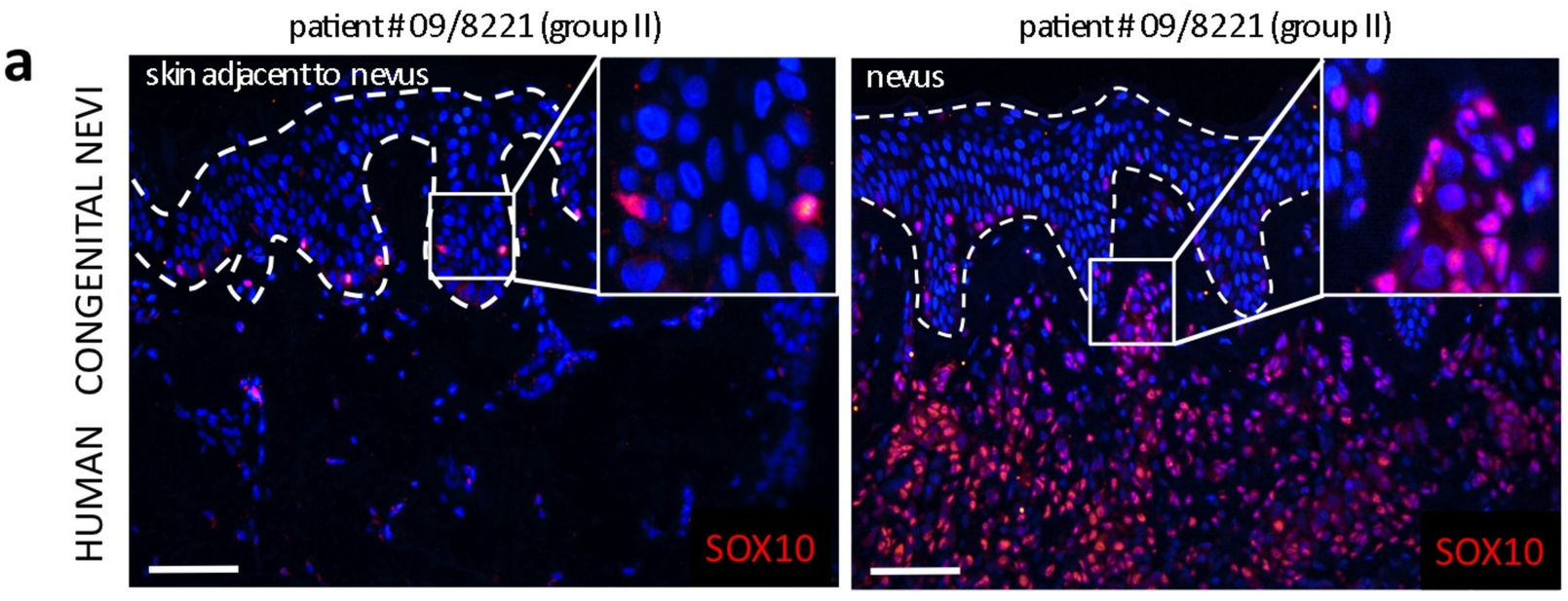

a
b
c
H&E
d
WT
Tyr::Nras^{Q61K}
P9
1 cm
e
SNOUT
PAW
WT
Tyr::Nras^{Q61K}
f
SG
H&E

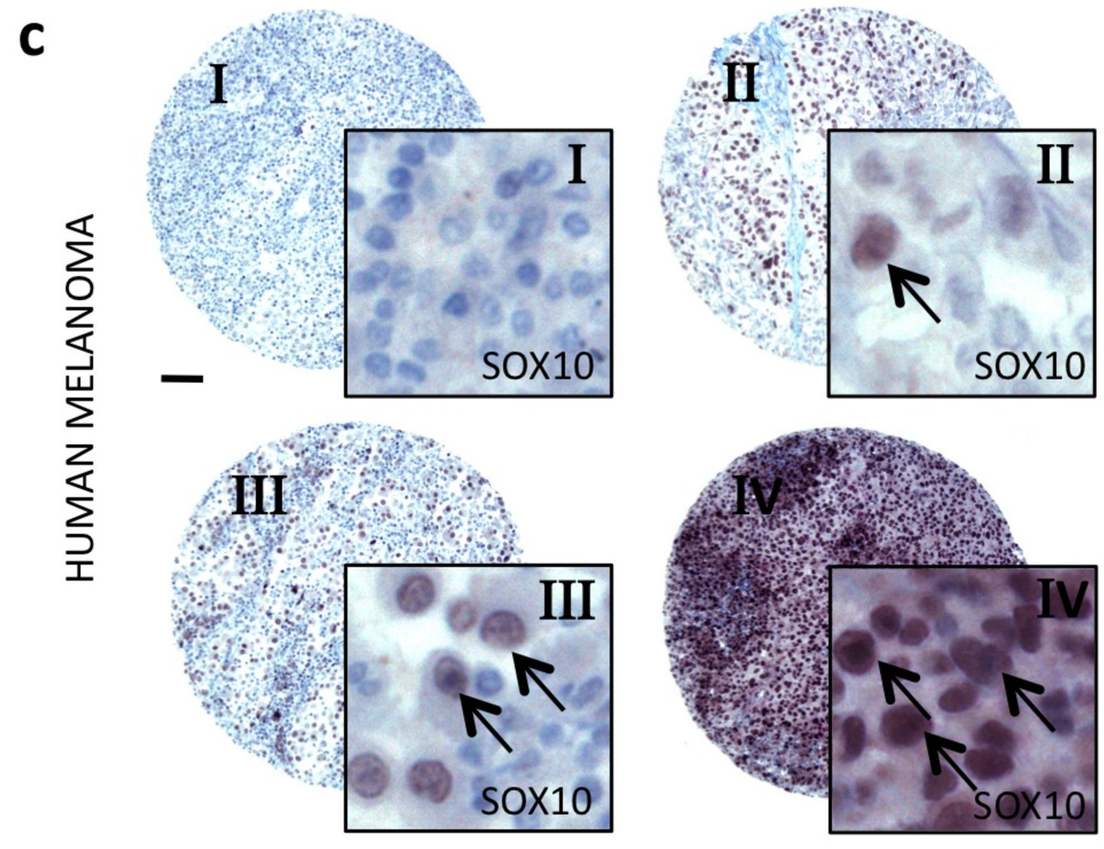
Figure1



b

Expression of SOX10 in human giant congenital nevi (CN)

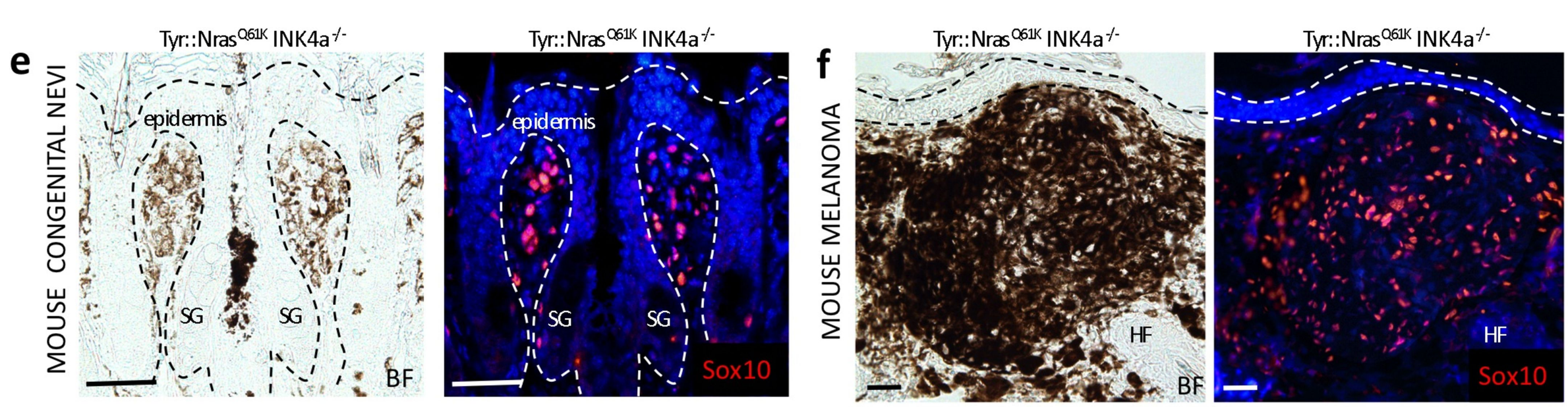
Specimen	Size (cm)	N=	SOX10+
CN group I	1.5-10	n=4	4/4
CN group II	11-20	n=9	9/9
CN group III	21-40	n=4	4/4
Total samples		n=17	17/17



d

Expression of SOX10 in human melanoma

Specimen	SOX10 positive cells (% of total tumor cells)			
	I (< 10%)	II (10 - 50%)	III (50 – 90%)	IV (>90%)
Primary melanomas (n=48)	O	7	8	85
		Σ=100 %		
Metastases (n=130)	13	3	12	72
		Σ=87 %		



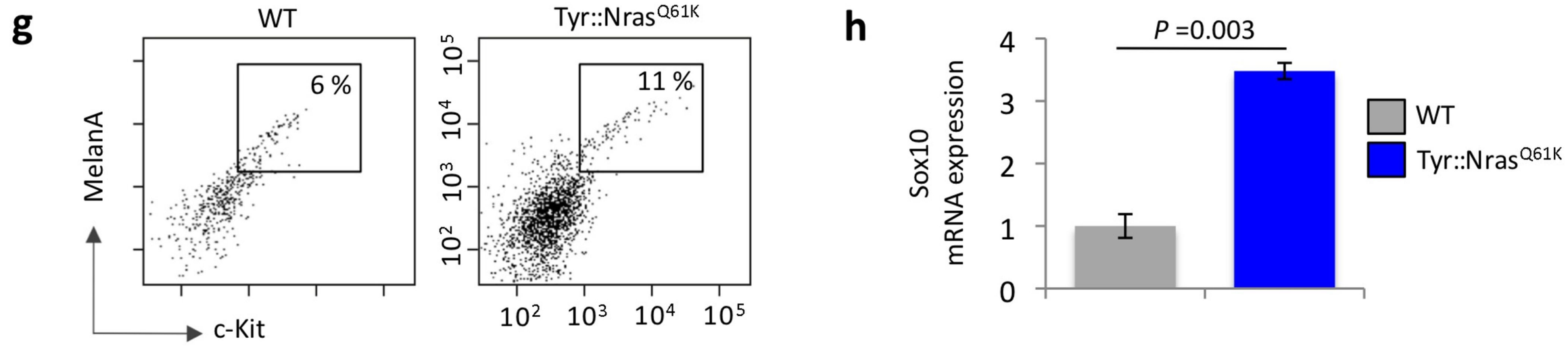
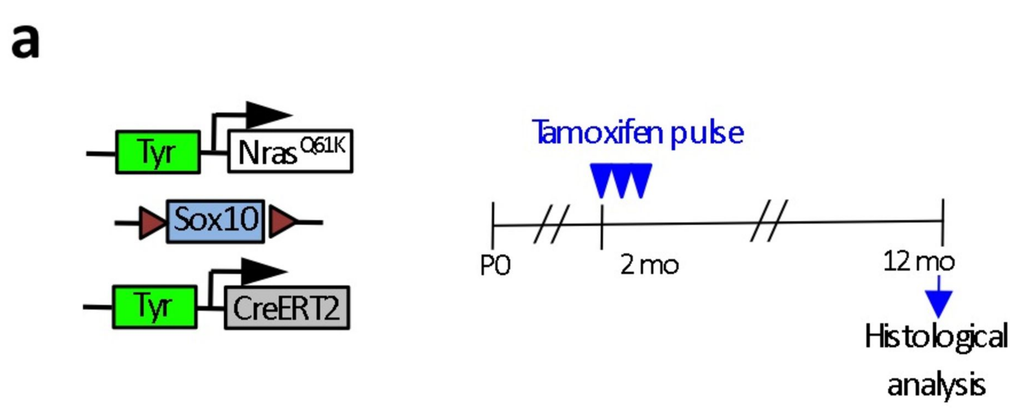
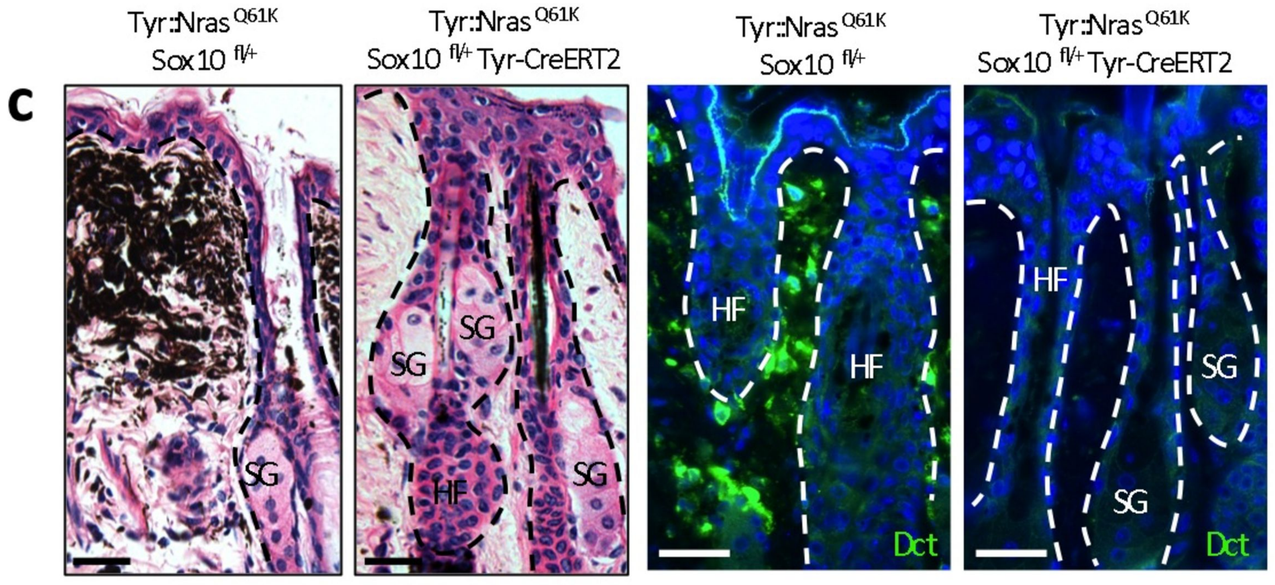
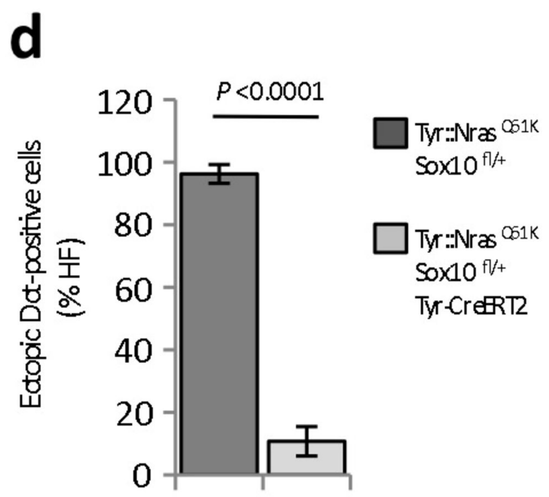


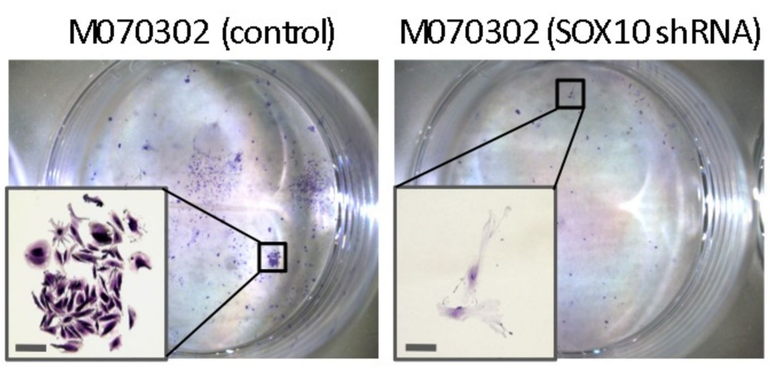
Figure 2

Figure 3



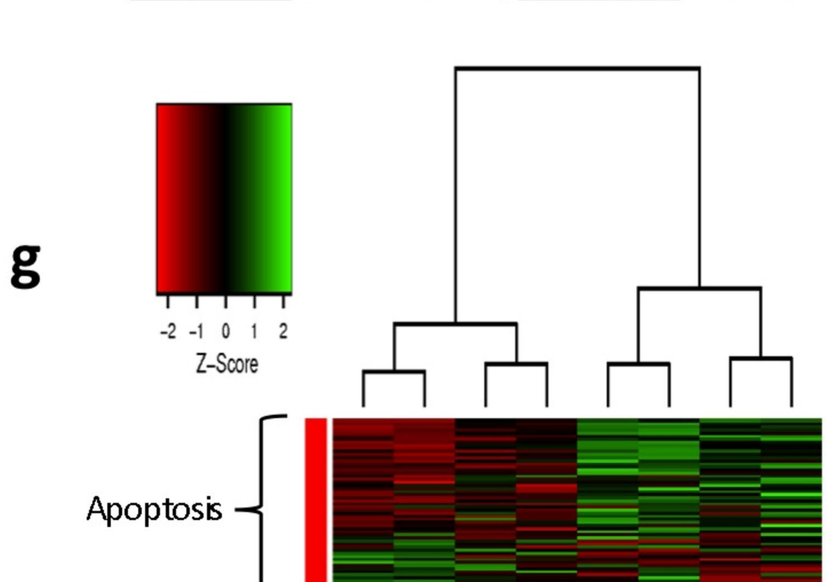


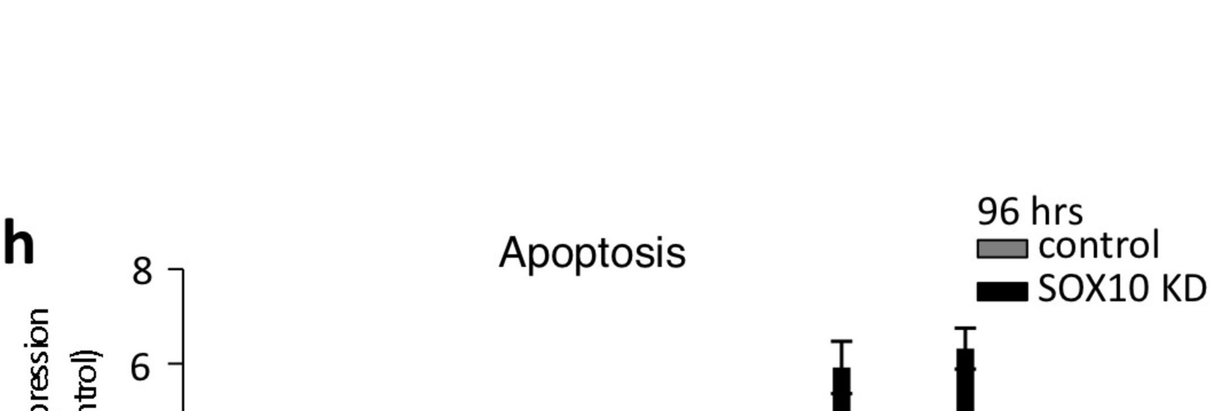




f *In vivo* tumor formation efficiency (subcutaneous injections)

Recipient mice	NOD/SCID	Nude		
Cell type	M010817	M010817	M070302	Summary
Control shRNA	3 (4)	3 (4)	4 (4)	11 (14)
SOX10 shRNA	0 (6)	0 (4)	0 (4)	0 (16)





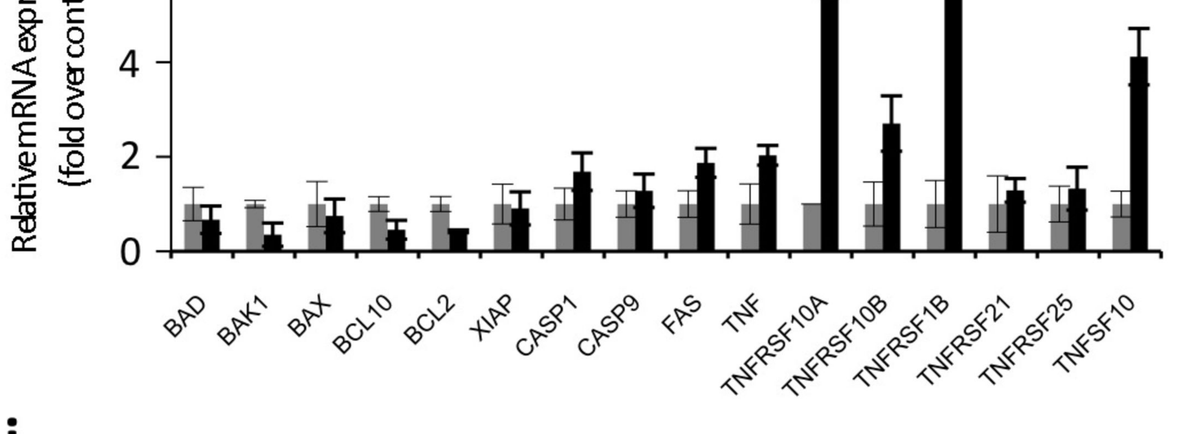


Figure 4

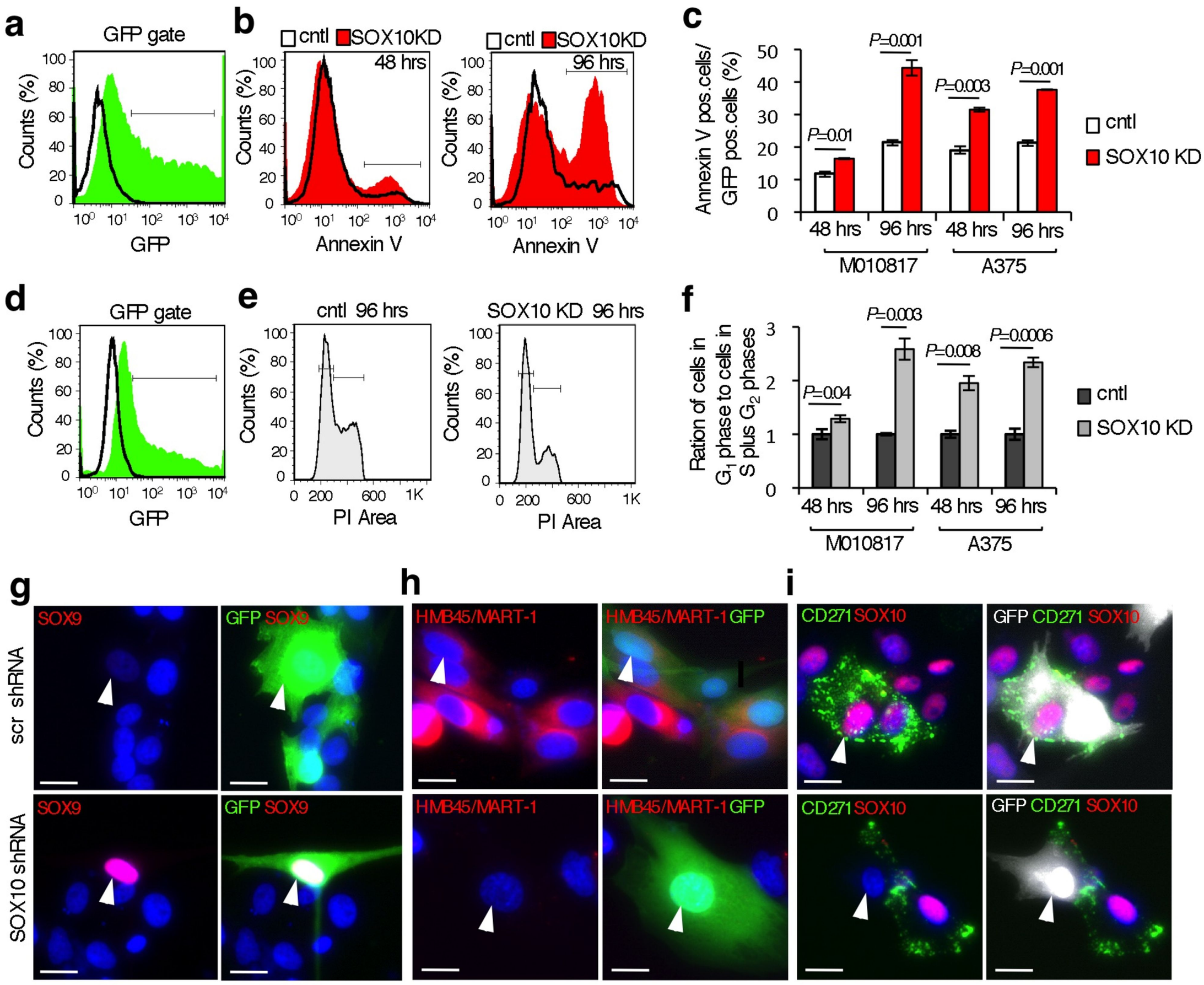

a
GFP gate
Counts (%)
GFP
b
cntl SOX10KD
48 hrs
96 hrs
Annexin V
c
Annexin V pos.cells/ GFP pos.cells (%)
P=0.01
P=0.001
P=0.003
P=0.001
cntl
SOX10 KD
48 hrs
96 hrs
M010817
A375
d
GFP gate
e
cntl 96 hrs
SOX10 KD 96 hrs
PI Area
f
Ration of cells in G1 phase to cells in S plus G2 phases
P=0.04
P=0.003
P=0.008
P=0.0006
g
h
i
SOX9
GFP SOX9
HMB45/MART-1
HMB45/MART-1 GFP
CD271 SOX10
GFP CD271 SOX10
scr shRNA
SOX10 shRNA
j
k
HMB45 / MART-1 pos cells / GFP pos cells (%)
P<0.001
control
SOX10 KD
l
CD271(p75NTR) pos cells / GFP pos cells (%)

Figure 5